

In males: heart (2.15 vs 1.91 g in controls), prostate (1.1 vs 1.42 g in controls), salivary gland (0.63 vs 0.81 g in controls), seminal vesicle (1.5 vs 1.9 g in controls). The relative heart weights at these high doses were increased by 8% and prostate weights decreased by 25%.

In females: brain (2.01 vs 2.09 g in controls), uterus (1.1 vs 0.69 g in controls), and ovaries (110 vs 90.9 g in controls). The relative uterus and ovary weights at these high doses were increased by 50% and 10% resp.

Gross pathology: An increased incidence of subcutaneous masses in mammary gland region and mammary gland cysts were observed in female rats (In both dead rats, and animals sacrificed at the end of study). Haemorrhagic depressions were observed in the stomach corpus mucosa in both sexes. The n=32/group includes both, the dead animals and those that survived till terminal sacrifice.

In females

Subcutaneous masses	7/32, 12/32, 15/32, 11/32, 7/32
Mammary gland cysts	6/32, 7/32, 5/32, 3/32, 9/32
Haemorrhagic depressions	0/32, 1/32, 4/32, 6/32, 8/32

In males

Haemorrhagic depressions	1/32, 2/32, 1/32, 10/32, 12/32
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Histopathology: The time-to-tumor method (Peto et al, 1980^a) was used to examine the number of animals with mammary tumors (MT). The incidence of animals with benign and malignant mammary gland tumors combined ($p=0.003$) or benign mammary gland tumors alone ($p=0.0039$) were significantly higher at 100 U/kg/bid of X14 in rats compared to controls. Malignant mammary tumors were also increased with 100 U/kg/bid of X14, but it was not significant ($p=0.090$). However, the incidences of these mammary tumors was not significantly increased in animals treated with HM(ge) at 100 U/kg/bid compared to controls (in a similar analysis). **Slight but not significant ($p=0.062$, two tailed comparison with HM(ge)) increases in the incidence of benign + malignant mammary gland tumors was observed with X14 compared to HM(ge) at 100 U/kg/bid.** No differences in total mammary gland tumors were noted between controls and 200 U/kg/day with X14 or HM(ge). Erosion of the glandular epithelium was noted in dead animals at 50 and 200 U/kg/day, these animals had died from hypoglycemia. Also focal seminiferous atrophy, often associated with vacuolation of sertoli cells was noted in the testis of male animals, although some of these were also noted in controls. The testicular changes with the drug may be due to disrupted lactate metabolism in sertoli cells by insulin-induced hypoglycemia, as developing spermatocytes are dependent on lactate as an energy source. Pituitary adenoma (benign) and/or hyperplasia was observed in all animals but these were not significantly different between control and with 100 U/kg/bid of X14 or HM(ge). Sponsor states that the increase in mammary tumors observed in this study was also noted in 2 previous studies with insulin or _____ in female rats (one was an exploratory study in female

rats, see toxicity summary, the data on the other study were not provided), and that these compounds cause mammary tumors in mammary prone female Sprague-Dawley CD rats. However, this study suggests that the incidence of mammary tumors with X14 may be higher than with human insulin, and further studies may be required to clearly establish its role in the induction of mammary gland tumors

^a = Peto R et al, In "long-term and short term screening assays for carcinogens: a critical appraisal. IARC Monographs on the evaluation of the carcinogenic risk of chemicals to humans. Supplement 2, page 311-426. International Agency for Research on Cancer, Lyon, 1980.

Doses (U/kg/day): 0, 10, 50, 200 of X14, and 200 of HM(ge)

Males

Stomach (erosion of stomach in dead rats)

Erosion of glandular epithelium 0/2, 1/1, 2/4, 8/18, 12/20

Testes (seminiferous tubular changes)

Focal seminiferous tubular epithelial atrophy 0/32, 0/1, 0/4, 4/32, 3/32

Vacuolation of sertoli cells 0/32, 0/1, 0/4, 2/32, 2/32

seminiferous tubular atrophy 4/32, 0/32, 0/4, 1/32, 1/32

Pituitary

Adenoma (benign) and/or hyperplasia 6/32, 0/1^b, 2/4^b, 7/32, 9/32

Females

Mammary tissue (n= 32/dose)

1. Rats bearing mammary tumors (benign+ malignant) 7, 11, 11, 11*, 6^c

2. Rats with benign tumors (fibroadenoma/adenomas) 6, 9, 10, 8**, 5

3. Rats with malignant tumors (adenocarcinomas) 2, 4, 2, 4***, 1

4. Rats with more than one MT 4, 2, 2, 3, 3

5. Total number of Mammary tumors 11, 13, 13, 14, 15

Stomach (erosion of stomach in dead rats)

Erosion of glandular epithelium 0/3, 0/4, 1/3, 4/18, 5/17

Pituitary

Adenoma (benign) and/or hyperplasia 6/32, 1/4^b, 0/3^b, 7/31, 8/32

* p= 0.003 compared to controls (using the trend test for all mammary tumors).

**p=0.0039 compared to controls (using the trend test for benign mammary tumors)

***p= 0.090 compared to controls (using the trend test for malignant mammary tumors)

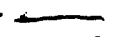
^b = At low-mid doses these were examined only in dead animals.

^cp=0.062- two tailed comparisons of X14 (200 U/kg/day) against HM(ge) at 200 U/kg/day.

Antibody Determination (study # 950183): Antibodies against X14 or HM(ge) were found in majority of all treated animals in both sexes in week 12 (7/10, 10/10, 9/10, 8/10

at 10, 50, 200 and 200 U/kg/day resp), but decreased in week 25 and 51 (1/10, 5/10, 5/10, 5/10). Antibody presence did not neutralize the effects of X14 or HM(ge), as no obvious effects on glucose were observed, i.e. hypoglycemic response was maintained throughout the study.

Toxicokinetics (study # 960270). Highest insulin X14 levels were noted at 1 hr (M+F 9.1-9.5, 132-187, 265-334 nM at 10, 50, and 200 U/kg/day resp) and 5 hrs. No gender differences were noted and linearity in Cmax and doses were observed. The rate of elimination of the drug did not increase with time, however no tabular data were provided, only figures were given (see appendix).

Conclusions: Treatment of the rats with the old X14 (at doses of 0, 5, 25, 100 U/kg/bid) and human insulin (HM(ge) 100 U/kg/bid) for 1-year caused dose related mortality in animals (deaths were 0, 2, 9, 32, and 35 in five above groups resp due to hypoglycemia, associated with empty GI contents and hemorrhagic depression/erosions in the stomach wall, which were noted in 0, 1, 3, 7, and 17 dead animals resp). Since more animals were dying at 200 U/kg/day in group 4 and 5, in week 25 in groups 4 and 5 the drug was reduced by 50% to 50 U/kg/bid. In week 27, all groups were changed from twice to once daily dose, and for groups 4 and 5, the daily dose was 100 U/kg/day. In week 38, in groups 4 and 5, the drug was further reduced to 75 U/kg/day. In both sexes the drug caused, clinical signs (hypoglycemic episodes), increase in food and water consumptions (by 10-20%), and biochemical changes (TG increased by 18-42% and 13-21% with X14 and HM(ge) at 100 U/kg/bid, plasma glucose conc decreased with all drug doses at 1-hr after dosing. Plasma glucose in the mid-high dose groups stayed low at all times, after first few days of dosing). The drug caused increased incidence of subcutaneous masses in mammary gland region (7, 12, 15, 11, & 7 at 0, 10, 50, 100 of X14 U/kg/bid or HM(ge) resp). Haemorrhagic depressions were observed in the stomach corpus mucosa in both sexes (1, 3, 5, 16 & 20 resp). Histopathology showed that the incidence of animals with benign + malignant mammary gland tumors (7, 11, 11, 11*, 6 at 0, 10, 50, 100 of X14 U/kg/bid or HM(ge) resp), or benign mammary gland tumors alone (6, 9, 10, 8*, and 5 resp) were significantly higher at 100 U/kg/bid of X14, compared to vehicle controls. These were not significantly increased at similar doses of HM(ge), compared to controls, and no significant differences in the incidence of mammary tumors was observed between X14 and HM(ge) at 100 U/kg/bid. The number of malignant tumors were higher with X14 at 100 U/kg/bid (4 vs 2 in vehicle controls, p=0.09), but not significantly increased. However no differences in total number of mammary gland tumors were noted between controls vs 200 U/kg/day of X14 or HM(ge), i.e. 14 and 15 resp vs 11 in controls. Sponsor states that the increase in mammary tumors with X14 seen here was also noted in 2 previous studies with insulin or  in female CD rats (NN study # X14/930308, and NN study # actrapid/940267), this suggests that these compounds cause mammary tumors in mammary prone female Sprague-Dawley CD rats (i.e. lobular hyperplasia with an increase in the number of acini in a mammary lobule in this strain, at this age). Also seminiferous tubular (ST) atrophy in testes of male animals was observed in both treated and controls, however focal ST epithelial

atrophy, associated with vacuolation of sertoli cells was only noted in the drug (or with human insulin) treated rats. The testicular changes with the drug may be due to disrupted lactate metabolism in sertoli cells by insulin-induced hypoglycemia, as developing spermatocytes are dependent on lactate as an energy source. Antibodies against X14 or HM(ge) were found in majority of treated animals in both sexes in week 12 (7/10, 10/10, 9/10, 8/10 at 10, 50, 200 and 200 U/kg/day resp), but decreased in week 25 and 51 (1/10, 5/10, 5/10, 5/10). Antibodies had no effects on the reduction of glucose levels or on hypoglycemic related deaths, suggesting that antibodies did not neutralize the effects of X14 or human insulin. The 'no toxic effect dose' could not be determined in this study, as deaths also occurred at lower doses of drug, due to insulin-induced hypoglycemia. The effects of X14 and human insulin in general were similar in this 1-year toxicity study in rats.

Four Week-Toxicity Studies in Dogs After Twice Daily Subcutaneous Injection, followed by a 4-week recovery period (Study No. 950379):

Sponsor's ID Study #: 950379

Amendment #, Vol #, and page # : volume 22, page 23

Conducting laboratory : _____

Date of study initiation: June 6, 1996

GLP compliance: Yes

QA Report: Yes (X) No (), Is the evaluation based on a final, QA report: Yes

Methods: This study examined the effects of the new drug (at 0.25, 0.5, and 1.0 U/kg/day) in dogs for 4 weeks. At the end of treatment period all groups were sacrificed, except the one animal/sex in the control and the high dose groups, which were kept for additional 4 week of drug free recovery period.

Dosing information:

species: Pure-bred Beagle dogs

#/sex/group or time point: 4/sex/group

age: 19-21 weeks old

weight: 5.9-9.7 kg.

satellite groups used for toxicokinetics or recovery: 1/sex/group were used for reversibility studies.

Dosage groups in administered units: Four groups (4 dogs/sex/group) were given either vehicle (0.15% phenol, 0.172% M-cresol and 1.60 % glycerol _____) or X14 (new process drug) twice daily by subcutaneous injections (on the dorsal side) at doses of 0.25, 0.5, 1.0 U/kg/bid for 4 weeks (or total dose of 0.5, 1.0, 2.0 U/kg/day).

Route, form, volume, and infusion rate (if i.v.): Subcutaneous injections were given twice daily, 4 hrs apart, at a volume of 0.02-0.05 ml/kg, for 4 weeks.

Drug, lot #: X14 new process drug, Batch # C96013, C96016 and C96015.

Formulation/vehicle: Vehicle was 0.15% phenol, 0.172% M-cresol and 1.60 % glycerol.

Times at which Observations are made:

Clinical signs: Daily

Body weights: Once a week prior to dosing, and every day during treatment before the subdosing on that day.

Food consumption: Daily.

Ophthalmoscopy: Before treatment and during week 4 of treatment, and at the end of recovery period, using a _____ ophthalmoscope. Pupils were dilated using a tropicamide _____ ophthalmic solution, prior to examination. Also a slit lamp biomicroscope was used to evaluate any unidentified lesions.

Electrocardiograms (ECG): Before treatment and during week 4 of treatment, and at the end of recovery period (each time before administration of the first dose). Standard limb leads I, II, and III were recorded. Visually any abnormalities of the electrical complexes were recorded along with heart rates

Hematology: Prior to the first dose, during week 4, and at the end of recovery period.

Clinical chemistry: Prior to the first dose, during week 4, and at the end of recovery period.

Urinalysis: Prior to the first dose, during week 4, and at the end of recovery period 16 hrs samples were collected (water was withheld from animals for 5 hrs).

Bone marrow: This was obtained from each animal by sternbral puncture, and smears were examined by modified Wright's stain.

Gross pathology: At sacrifice.

Organs weighed: Highlighted in histopathology table

Histopathology:* At sacrifice, tissues from all animals were examined. Liver sections were stained with Oil-O-Red O, and with periodic acid Schiff reagent (PAS) for glycogen.

Toxicokinetics: Days 1 and 28, at 0, 1, 4, 5, and 8 hr (Study # 960452).

Plasma glucose profiles: Days 1 and 28, at 0, 1, and 4 hrs after first dose, 1 and 4 hrs after second dose.

Antibody determination: Prior to dosing, in week 4, and at the end of recovery period.

Results:

Mortality and Clinical Signs: No drug related mortalities were observed. One female in the high dose group (#210) was found extremely quiet on day 14, but a diagnostic test (biochemistry and hematology tests) did not reveal the illness. No incidences of hypoglycemia were recorded at any drug doses.

Body Weight and Food Consumption: No drug related effects on body weights were observed. Mean food consumptions were slightly reduced in high doses females by 5%. Again, one high dose female (#210) did not consume sufficient food on 13 occasions.

Bone Marrow: No drug related effects were observed.

Hematology: In week 4, lymphocytes were decreased in females at all doses _____ vs 5.6 in controls). No other drug related effects were observed.

Biochemical Parameters: In week 4, calcium values were decreased in females at mid-high doses (5.6 vs 6.0 in controls), and TG values were decreased at all dose (— vs 40 mg/dl, it was significant at a high dose). Combined data for male + females showed decreases in gamma glutamyltransferase (<1 vs <2 in controls), and in calcium (5.7 vs 6.0). No other drug related effects were observed. Urinary analysis showed significant decreases in urinary sodium in females in week 2 — vs 9.3 in controls), but not in week 4.

Plasma glucose concentration: These were reduced at 1 hr after each dose, and there was a dose dependent effect, 4 hrs after each dose normoglycemia was generally seen. The degree of suppression for some treated females was slightly greater on day 28 vs day 1.

Ophthalmic Examinations: No drug related effects were observed.

ECG: No drug related effects were observed, including on heart rates.

Organ Weights: Relative adrenal weights in females were slightly increased (1.1, 1.3, 1.3, 1.4 g resp in 4 groups).

Pathology: No drug related effects were observed, except subcutaneous hemorrhage at injection sites in all groups, including controls. There were no other dosage or treatment related macroscopic or microscopic pathological changes.

Antibody Determination: Antibodies against X14 were found in all treated groups after 4 weeks. The frequency of antibody positive animals appeared to correlate to the given dose (1/6, 1/6, and 2/8 animals had the presence of antibodies at 0.25, 0.5 and 1.0 U/kg/bid resp). Both recovery dogs (at high dose n=1/sex) were not antibody positive at the end of the study. However, these animals did not have antibodies after 4 weeks of treatment with the drug at the beginning of the recovery period, either.

Toxicokinetics (Study # 960452). Highest insulin X14 levels (pM) were generally noted at 1 and 5 hrs. These at 1 hr were as follows: Dose linearity was noted in both sexes.

	males	females (at 0, 0.25, 0.5, and 1.0 U/kg/bid)
Day 0	180, 195, 337, 740,	175, 185, 323, 709
Day 28	123, 168, 292, 940	79, 200, 344, 940

Conclusions: All dogs tolerated the new X14 well at doses of 0.25, 0.5 and 1.0 U/kg/bid for 4 weeks. Mean food consumptions were slightly reduced in high dosed females by 5%. Antibodies were found in all treated animals after 4 weeks of treatment, which correlated with the dose. There was a dose-dependent effect on blood glucose reductions at 1 hr; 4 hrs after each dose normoglycemia was generally seen.

**Three-Month Subcutaneous (Once Daily Injection) Toxicity Study of X14 in Dogs
(Study No. SX12660):**Sponsor's ID Study # — 12660Amendment #, Vol #, and page # : volume 22, page 311Conducting laboratory : _____Date of study initiation: Not givenGLP compliance: YesQA Report: Yes (X) No (), Is the evaluation based on a final QA report: YesMethods: This study examined the effects of the drug (at 1 and 4 IU/kg/day) in dogs for 3-months, control dogs received the vehicle only.**Dosing information:**species: Beagle dogs#/sex/group or time point: 4/sex/groupage: 4-months of ageweight: ≈6 kg.satellite groups used for toxicokinetics or recovery: N/A.Dosage groups in administered units: Three groups (4 dogs/sex/group) were given either vehicle (_____ medium, m-cresol) or old process drug X14 once daily by subcutaneous injections at doses of 1.0 and 4.0 U/kg/day for 90 days.Route, form, volume, and infusion rate (if i.v.): Subcutaneous injections were given once daily, at a volume of 0.1 ml/kg, for 3-months.Drug, lot #: X14 old process drug, Batch # P-10691 _____, P-10791 _____Formulation/vehicle: medium for Actrapid m-crestol.**Times at which Observations are made:**Clinical signs: Twice dailyBody weights: Weekly.Food consumption: Daily, and any unconsumed food left was weighedOphthalmoscopy: Before treatment and before termination of the study, using _____ ophthalmoscope. Pupils were dilated using a tropicamide _____ ophthalmic solution, prior to examination. Also a slit lamp biomicroscope was used to evaluate any unidentified lesions.Hematology: Prior to dosing and on day 90Clinical chemistry: Prior to dosing and on day 90.Urinalysis: Prior to the first dose, and on day 89-90. 24 hrs samples were collected .Gross pathology: At sacrifice.Organs weighed: Highlighted in histopathology tableHistopathology:* At sacrifice, tissues from all animals were examined.**Results:**

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Mortality and Clinical Signs: One female dog at 4 U/kg dose died due to hypoglycemic episode, on day 56, histopathology showed alveolar hemorrhage and perivascular edema in lungs and diffuse subendocardial hemorrhages in heart. These hypoglycemic episodes were noted at 1 U/kg of X14 (in $\frac{3}{4}$ of males and $\frac{1}{4}$ of females), and at 4 U/kg of X14 (in $\frac{2}{4}$ males and $\frac{3}{4}$ females), the number of days these animals had episodes was increased at a higher dose. In order to avoid the hypoglycemic episodes, when dogs ate less than 100 g of the diet, they were not dosed. In group 2 (1 IU/kg) and group 3 (4 IU/kg), the mean number of days that dogs were not dosed were 3 and 11 days resp.

Body Weight and Food Consumption: No drug related effects on body weights were observed. No data on food consumptions were provided

Hematology: At 3-months, at 1 and 4 U/kg X14, the Hb (9.5, & 9.5 vs 10.3 in controls) and PCV (44.3 & 44.5 vs 48 in controls) were decreased in males.

Biochemical Parameters: In males at high dose inorganic phosphorous (2.0 vs 2.3 in controls) and α_1 -globulin (10.1 vs 12.3 in controls) were decreased, while α_2 -globulin was increased in males at both doses (— vs 11.0 in controls). Both α_1 — vs 12.2 in controls) and α_2 (12.5 vs 10.0 in controls) were also similarly altered in females at 3-months. These effects on globulin may be due to immunologic effects of X14. No drug related effects on urine analysis were observed

Ophthalmic Examinations: No drug related effects were observed.

Organ Weights: No drug related effects were observed.

Pathology: No drug related effects were observed, except subcutaneous fibrosis and hemorrhage at injection sites were noted in all groups, including in controls. There were no other dosage or treatment related macroscopic or microscopic pathological changes.

Conclusions: All dogs tolerated the X14 well at doses of 1.0 U/kg/day for 3 months. Hypoglycemic episodes were noted at both 1 and 4 U /kg/day (with higher incidences at 4 U/kg/day). At 4 U/kg/day, 1 of 4 female dogs died due to hypoglycemia. Since the hypoglycemic episodes increased at higher dose, when dogs eat less than 100 g of the diet, they were not dosed, at 1 U/kg and 4 U/kg, the mean number of days that dogs were not dosed were 3 and 11 days resp.

One Year Subcutaneous (Twice Daily Injection) Toxicity Study of X14 in Dogs (Study No. 940302):

Sponsor's ID Study #: 940302

Amendment #, Vol #, and page # : volume 23, page 1

Conducting laboratory : _____

Date of study initiation: October 6, 1994

GLP compliance: Yes

QA Report: Yes (X) No (), Is the evaluation based on a final, QA report: Yes

Methods: This study examined the effects of the old drug (at 0.25, 0.5, and 1.0 U/kg/bid) in dogs for 52 weeks. Also a group with recombinant human insulin HM(ge) at 1 IU/kg/bid for 52 weeks, was included for comparative purposes.

Dosing information:

species: Pure-bred Beagle dogs

#/sex/group or time point: 4/sex/group

age: 18-23 weeks old

weight: 6.8-10.9 kg.

satellite groups used for toxicokinetics or recovery: N/A.

Dosage groups in administered units: Four groups (4 dogs/sex/group) were given either vehicle (0.15% phenol, 0.172% m-cresol and 1.60 % glycerol _____ adjusted to pH 7.4 with NaOH) or X14 twice daily by subcutaneous injections (on the dorsal side) at doses of 0.25, 0.5, 1.0 U/kg/bid for 52 weeks (or total dose of 0.5, 1.0, 2.0 U/kg/day). The fifth group received recombinant human insulin HM(ge) at 1 IU/kg/bid for 52 weeks, for comparative purposes. Since there were increased incidences of hypoglycemic episodes in dogs, in week 29 (day 1), all animals received the full dose of the drug once daily (instead of twice daily) at 10 AM.

Route, form, volume, and infusion rate (if i.v.): Subcutaneous injections were given twice daily on the dorsal side, 4 hrs apart, at a volume of 0.02-0.10 ml/kg, for 52 weeks.

Drug, lot #: X14 old process drug, Batch # 6794 (conc _____), 6994 (conc _____), Batch # for HM(GE) 6094 (_____).

Formulation/vehicle: Vehicle was 0.15% phenol, 0.172% m-cresol and 1.60 % glycerol.

Times at which Observations are made:

Clinical signs: Daily

Body weights: Once a week prior to dosing, and every day during treatment before the subdosing on that day.

Food consumption: Daily, and any unconsumed food left was weighed.

Ophthalmoscopy: Before treatment and during weeks 13, 26, 39 and 52 of treatment, using a _____ ophthalmoscope. Pupils were dilated using a tropicamide _____ ophthalmic solution, prior to examination. Also a slit lamp biomicroscope was used to evaluate any unidentified lesions.

Electrocardiograms (ECG): Before treatment and during weeks 13, 26, 39 and 52 of treatment (each time before administration of the first dose). Standard limb leads I, II, and III were recorded. Visually any abnormalities of the electrical complexes were recorded along with heart rates

Hematology: Prior to the first dose, during weeks 13, 26, 39 and 52.

Clinical chemistry: Prior to the first dose, during weeks 13, 26, 39 and 52.

Urinalysis: Prior to the first dose, during weeks 13, 26, 39 and 52, 16 hrs samples were collected (water was withheld from animals for 5 hrs).

Bone marrow: This was obtained from each animal prior to necropsy, by sternbral puncture, and smears were examined by modified Wright's stain.

Gross pathology: At sacrifice.

Organs weighed: Highlighted in histopathology table

Histopathology:* At sacrifice, tissues from all animals were examined. Liver sections were stained with Oil-O-Red O, and with periodic acid Schiff reagent (PAS) for glycogen.

Toxicokinetics (Study # 960130): Days 1, 28, week 13 and 26 at 0, 1, 4, 5, and 8 hr. In week 29, on day 1 and on one day in week 52, at pre-dose, 1, 4, 6 and 8 hrs. Plasma for X14, human insulin and endogenous canine insulin was assayed by _____

Plasma glucose profiles: Days 1 and 28, and during weeks 13 and 26 at 0, 1, and 4 hrs after first dose, 1 and 4 hrs after second dose. In week 29, on day 1 and in week 52, at pre-dose, 1, 4, 6 and 8 hrs

Other (antibody determination, study # 950223): Prior to dosing, in weeks 13, 26, 39 and 52. These were analyzed using _____ insulin X14 and human insulin.

Results:

Mortality and Clinical Signs: One male dog at 1 U/kg/bid dose was sacrificed (humane reasons, it suffered an irreversible neurological impairment) due to hypoglycemic episode, in week 27 (on day 6), histopathology showed ulceration in the esophagus, which was not related to treatment but contributed to poor condition. Similarly one of 4 females from the HM(ge) group was also sacrificed due to hypoglycemic episode, in week 20 (on day 6). These hypoglycemic episodes were noted with all doses of X14 and with HM(ge). In order to avoid the hypoglycemic episodes, when dogs ate less food (≈ 70 g of food), they were not dosed. The hypoglycemic episodes often required treatment with dextrose saline and/or glucagon and sometimes additional food. Scabs at injection site were observed in all animals including in controls. Some dogs showed clinical signs of hypoglycemia (incoordination, subdued behavior, twitching, trembling, collapse or convulsive-like behavior)

Body Weight and Food Consumption: One dog on HM(ge) who died had gained more weight prior to death due to increased food utilization, an adaptation to repeated hypoglycemia. No other drug related effects on body weights or on food consumptions were observed.

Bone Marrow: No drug related effects were observed on cellularity, cell distribution and morphology.

Hematology: No drug related effects were observed.

Biochemical Parameters: From week 26 onwards urea nitrogen values were decreased in all treated male dogs (— vs 20 in controls in week 52), these were not altered in females. No other significant drug related effects were observed.

Urine Analysis: Significantly decreased urinary protein was noted with — in males, but not in females at mid and high doses. At 52 weeks values were: males 146, 38, 28*, 29*, 59, females 36, 30, 21, 23, 58* mg/dl resp in 5 groups (*p<0.05 by William's test in females and by Dunnetts test in males)

Plasma glucose concentration: These were reduced at 1 hr after each dose, and there was a dose dependent effect, 4 hrs after each dose normoglycemia was generally observed. In week 13/26 the glucose suppression was slightly greater than on day 28. However in week 39/52, the individual animal variation was noted to the dose, in some dogs the degree of suppression was not as marked as in week 29 and normoglycemia was achieved earlier.

Values at 0, 0.25, 0.5, 1.0 of X14 U/kg/bid, and HM(ge) 1 U/kg/bid resp:

Plasma glucose conc at 1 hr	Males	Females
Day 1	6.8, 5.7, 3.8, 3.2, 6.9	6.7, 5.7, 4.8, 2.9, 3.4
Day 28	6.3, 5.2, 3.4, 2.5, 2.9	6.5, 4.6, 3.7, 2.5, 3.2
Week 13	6.3, 4.1, 2.6, 2.5, 2.9	6.5, 4.0, 2.7, 2.3, 3.0
Week 26	5.7, 4.2, 2.8, 2.4, 2.7	5.9, 3.5, 3.6, 1.9, 3.6
Week 29	6.2, 3.1, 2.3, 2.1, 2.6	5.8, 2.7, 2.8, 2.3, 2.7
Week 39	5.9, 2.9, 2.4, 2.2, 2.0	5.3, 2.6, 2.8, 2.3, 2.4
Week 52	5.4, 3.1, 2.1, 2.2, 1.9	5.2, 2.5, 2.4, 1.5, 2.1

Ophthalmic Examinations: No drug related effects were observed.

ECG: No drug related effects were observed, including on heart rates.

Organ Weights: No drug related effects were observed. Pituitary weights were significantly increased in males at 1 U/kg/bid of X14 (99 vs 73 g in controls). Uterus weights were decreased in all treated females (— vs 28.9 g in controls). these were not associated with any histopathology

Pathology: No drug related effects were observed, except subcutaneous hemorrhage at injection sites were noted in all groups, including in controls. There were no other dosage or treatment related macroscopic or microscopic pathological changes.

Antibody Determination (Study # 950223): Antibodies against X14 and human insulin were found in very few animals and in low amounts. The antibody positive animals appeared to be very low (in week 52 the number of animals that had the presence of antibodies were as follows: males 0/4, 1/4, 0/4, 0/4, females 0/4, 0/4, 0/3, 1/3 at 0.25, 0.5, 1.0 U/kg/bid of X14 and 1.0 U/kg/bid of HM(ge) resp) .

some had lachrymation. All iv doses produced convulsions in rats and mice, but animals recovered.

In a 28-day subcutaneous toxicity study in rats, animals received the old process drug at 0, 12.5, 50, and 200 U/kg/day (once daily). Clinical signs (reddening, swelling, weeping, ulcerations, encrustation or bleeding) were observed at the injection site. At 200 U/kg/day, the incidence of periadipocyte hepatocyte vacuolation was higher in males. No other treatment related histopathological changes were observed.

In a 28-day subcutaneous toxicity study, with a 28-day recovery period in rats, animals received the new drug (at 5, 25, and 100 U/kg/bid or total doses of 10, 50 and 200 U/kg/day), as well as the old drug (at 100 U/kg/bid) for comparison purposes. This was because a change in manufacturing process was implemented in the drug development

_____ The later drug manufacturing process was designated as the new drug. Highest insulin X14 levels were noted at 1 and 5 hrs, and no significant differences were noted with new vs old X14. All doses produced hypoglycemia in animals, and recovery in glucose levels occurred quite rapidly in the lower drug groups, but more slowly at higher doses. All animals tolerated the new X14 (at total doses of 10, 50 and 200 U/kg/day) and old X14 (200 U/kg/day) for 4 weeks, except in thyroids, ectopic thymic tissue was noted in 1 and 3 of 10 males, and 2 and 2 of 10 females at 200 U/kg/day of new and old drug resp (vs none in controls). Pharmacological response was associated with one instance of hypoglycemic death at the high dose with increase in body weight and food consumption (by up to 13%). Antibodies were found in all treated animals after 4 weeks of treatment, which correlated with the dose and increases in the titer. Antibodies were also found after the 4-week drug free recovery periods. The NOAEL dose of X14 (the new process drug) in this 1-month rat study was 50 U/kg/day.

In a 3-month subcutaneous toxicity study in rats, doses of 0, 12.5, 50, and 200 U/kg/day of old process X14 (once daily) were used. Twelve rats died at 200 U/kg/day due to insulin induced hypoglycemia. High dosed females had treatment related increase in body weight and body weight gain. Serum protein levels were dose-dependently low in all drug treated female rats. Changes at the injection site were attributable to _____ (m-cresol). The tolerated dose of X14 (the old process drug) in this 3-months rat study was 50 U/kg/day.

In a 1-year exploratory subcutaneous toxicity study in female rats (study # 930803, a draft report), effects of X14 (at 0, and 200 U/kg/day of old process, once daily) were compared with _____

_____ as well as with human insulin (Actrapid at 200 U/kg/day), to investigate the incidence of mammary tumors in female _____ Sprague-Dawley CD rats with the drug. 3, 5 and 8 of 20 rats died with X14, _____ and human insulin resp at 200 U/kg/day due to insulin induced hypoglycemia. In all groups, tumors in mammary glands were found to be of the same type, all groups had hyperplasia of mammary glandular epithelial cells, with no significant differences in the severity vs the human

insulin. The majority of mammary gland tumors were benign and classified as fibroadenomas. The number of rats that had benign tumors were 4/20, 7/18, 11/17 and 8/17 in controls, X14, — and Actrapid resp (the malignant tumors in these groups were 1/20, 4/18, 3/17 and 3/17 resp). Combined analysis (by Peto et al) of total benign + fatal adenomas indicated that — was associated with higher incidence of tumors ($p < 0.01$), actrapid was also positive compared to control ($p < 0.05$). The total number of benign mammary tumors (multiple tumors counted per rat) were 6/20, 11/18, 26/17 and 11/17 in controls, X14, — and Actrapid resp. No treatment related changes in pituitary weights were observed, but pituitary gland adenomas were 1/20, 3/18, 3/15, and none resp, in above 4 groups. Thus no pituitary tumors were observed with regular human insulin but slight increases in these were found with — (X14 and —). Hormone producing pituitary gland adenomas have been shown to induce mammary gland tumors (Greaves and Faccini, 1992). This preliminary study demonstrated that the incidence and the onset of palpable masses in — group were significantly higher when compared with X14/human insulin or controls. The tumorigenic potential of X14 was not significantly greater than endogenous insulin.

In a 1-year toxicity study in rats (study # 940301), the effects of the old drug X14 (at 0, 5, 25 & 100 U/kg/bid) vs with recombinant human insulin (HM(ge) at 100 U/kg/bid) were compared. Since animals were dying at 100 U/kg/bid (or total dose of 200 U/kg/day) in groups 4 and 5, in week 25 the daily dose was reduced to 100 U/kg/day, and all groups were changed from twice to once daily dose. In week 38, in groups 4 & 5 the drug was reduced to 75 U/kg/day. Highest insulin X14 levels were generally noted at 1 and 5 hrs in rats, and dose linearity was noted in both sexes with no gender differences. Drug related mortality increased with increase in dose (deaths were 0, 2, 9, 32, and 35 in five above groups resp due to hypoglycemia, associated with empty GI contents and hemorrhagic depression/erosions in the stomach wall). In both sexes the drug caused, clinical signs (hypoglycemic episodes), increased food and water consumptions (by 10-20% at 25 and 100 U/kg/bid), biochemical changes (TG increased by 18-42% and 13-21% with X14 and HM(ge) at 100 U/kg/bid, plasma glucose conc decreased with all drug doses at 1-hr after dosing), increased incidence of subcutaneous masses in mammary gland region in females (7, 12, 15, 11, & 7 at 0, 5, 25, 100 U/kg/bid of X14 and 100 U/kg/bid of HM(ge) resp). Also haemorrhagic depressions were observed in the stomach corpus mucosa in both sexes (1, 3, 5, 16 & 20 in five groups resp). Histopathology showed that the incidence of animals with benign + malignant mammary gland tumors combined (7, 11, 11, 11*, 6 at 0, 5, 25, 100 U/kg/bid of X14 and 100 U/kg/bid of HM(ge) resp), or benign mammary gland tumors alone (6, 9, 10, 8*, and 5 resp, * $p = 0.003$) were significantly higher in treated animals (at 100 U/kg/bid of X14), compared to vehicle controls. However, lower number of tumors were noted in animals with HM(ge), and these were not significantly different from controls ($p = 0.2$). **Slight but not significant ($p = 0.062$) increases in the incidence of benign + malignant mammary gland tumors was observed with X14 compared to HM(ge) at 100 U/kg/bid.** No differences in total number of mammary gland tumors were noted between controls vs 200 U/kg/day of X14. Pituitary adenomas (benign)/hyperplasia were not significantly different between control and with 100 U/kg/bid of X14 or HM(ge)

in this study. Also seminiferous tubular (ST) atrophy in testes of male animals was observed in both treated and controls, however focal ST epithelial atrophy, associated with vacuolation of sertoli cells was only noted in the drug treated rats (at 100 U/kg/bid of X14 or HM(ge)). The testicular changes with the drug may be due to disrupted lactate metabolism in sertoli cells by insulin-induced hypoglycemia, as developing spermatocytes are dependent on lactate as an energy source. Antibodies against X14 or HM(ge) were found in majority of treated animals in both sexes in week 12 (7/10, 10/10, 9/10, 8/10 at 10, 50, 200 and 200 U/kg/day resp), but decreased in week 25 and 51 (1/10, 5/10, 5/10, 5/10). In general similar effects were observed with both X14 and regular human insulin at high doses. Sponsor states that the increase in mammary tumors was also noted in 2 previous studies with insulin or _____ in female rats, suggesting that these compounds cause mammary tumors in mammary prone Sprague-Dawley CD rats. However _____, clearly had the potential to promote mammary gland tumors (in 1-year exploratory studies in rats), and it is possible that _____ or X14) may have higher potential than regular human insulin (or differences in onset of tumors), and further studies with X14 may be required to establish its role. It is interesting to note that in the exploratory study the survival of animals was better with 200 U/kg/once a day, than when 200 U/kg dose was given bid (in a 1-year tox study). In the regular 1-year study, more animals were dying at high doses, and doses had to be reduced from 100 U/kg/bid (total dose 200 U/kg/day) to 100 U/kg once a day in week 25, and then to 75 U/kg/once a day in week 38, these discrepancies may explain the final differences in the tumor findings in the above two studies (exploratory and regular 1-year study). In summary, these studies suggest that 200 U/kg/day (approximately 32 times the recommended maximum human dose) slightly (but not significantly) increased the incidence of benign with malignant tumors combined, in female rats with X14, from those observed with regular human insulin. The relationship of these findings to humans is unclear. The tolerated doses of the drug could not be determined here, as deaths occurred even at low doses of the drug, due to consistent long-term hypoglycemia.

In a 28-day subcutaneous toxicity study, with a 28-day recovery period in dogs, animals received the new drug at doses of 0.25, 0.5, and 1.0 U/kg/bid (or total doses 0.5, 1.0 and 2.0 U/kg/day). Highest insulin X14 levels were generally noted at 1 and 5 hrs in dogs, and dose linearity was noted in both sexes. Mean food consumptions were slightly reduced in high dosed females by 5%. Antibodies (in 1/6, 1/6, and 2/8 animals at 0.25, 0.5 and 1.0 U/kg/bid resp) were found in all treated animals after 4-weeks of treatment and correlated with the dose, the recovery dogs (at high dose n=1/sex) were antibody negative but they were negative to start with at the end of the dosing period. Plasma glucose conc were reduced at 1 hr after each dose, and there was a dose dependent effect, 4-hrs after each dose, normoglycemia was generally seen. The NOAEL dose of X14 (the new process drug) in this 1-month dog study was 2 U/kg/day.

In a 3-month subcutaneous toxicity study in dogs, doses of 1.0 and 4.0 U/kg/day of old process X14 (once daily) were used. Hypoglycemic episodes were noted at both 1 and 4 U/kg/day doses of the drug. Since the hypoglycemic episodes increased at

higher dose, when dogs eat less than 100 g of the diet, they were not dosed. At 1 U/kg and 4 U/kg, the mean number of days that dogs were not dosed were 3 and 11 days resp. At 4 U/kg/day, 1 of 4 female dogs died due to hypoglycemia. Except some signs of subcutaneous fibrosis and hemorrhages at injection site, no other signs of toxicity were observed.

In a 1-year subcutaneous toxicity study in dogs, doses of 0.25, 0.5 and 1.0 U/kg/bid of old process X14, or 1.0 U/kg/bid of HM(ge) were used. The Highest drug (insulin X14) levels were generally noted at 1 hr. Dose linearity was noted in both sexes, and no sex differences were observed in TK, and no change in elimination over the year. All dogs tolerated the drug (X14) well, for 52 weeks. Hypoglycemic episodes were noted at all doses of the drug. At 1 U/kg/bid of X14 (1 of 4 male dogs) or human insulin (1 of 4 female dogs died) deaths were seen due to hypoglycemia. Very few animals had the presence of low levels of antibodies with X14 or human insulin (males 0/4, 1/4, 0/4, 0/4, females 0/4, 0/4, 0/3, 1/3 at 0.25, 0.5, 1.0 U/kg/bid of X14 and 1.0 U/kg/bid of HM(ge) resp.) .

In Immunotoxicity studies, X14 was less immunogenic than porcine or bovine insulin in rabbits (when 120 µmol was injected into rabbits twice a week, and serum insulin antibody binding was determined). In the transgenic mice (which were transfected with human insulin gene), neither X14 or human insulin elicited antibody formation, suggesting X14 has an immunogenic potential similar to human insulin.

Immunogenicity studies in 1-month rat and dog toxicity studies indicated a dose related correlation, with increased titers with increased doses and were also observed during the 1-month drug free recovery period in animals. However in longer immunogenicity studies (in 1-year rat and dog toxicity studies), a low titer in few animals was observed.

REPRODUCTIVE TOXICOLOGY:

Study title: Segment I/II. Effects of Subcutaneous X14 (Twice Daily) On Male/Female Fertility and Embryo-Fetal Development in Rats (Study # 940303)

Study No: 940303

Site and testing facility: _____

GLP compliance: Yes

QA- Reports Yes (X) No ():

Lot and batch numbers: Old process X14 06794 , _____ , 06994 (_____), 07194
_____ HM(ge) 06294 _____

METHODS:

Species/strain: Sprague-Dawley rats (CrI:CD BR VAF/plus strain), males 9-10 week old, 259-298 g, females 5-6 weeks old, 129-160 g .

Doses employed: 0, 5, 25 and 100 U/kg/bid (or total doses 0, 10, 50 and 200 U/kg/day)

Route of Administration: Subcutaneous

Number of animals/sex/dosing group: 24

Study Design: This study examined the effects of X14 on fertility and embryo fetal development in rats. Also a group was included to receive recombinant human insulin HM(ge) at 100 U/kg/bid for comparative purposes. Four groups of 24 male and female rats were given the three doses of the drug (at 0, 5, 25, and 100 U/kg/bid), or HM(ge) subcutaneously, twice daily (4 hrs apart). Males and females were treated for 4 and 2 weeks resp prior to mating, and throughout the mating. Males continued to be treated till termination of all females (till day 20 of gestation). Females continued treatment till day 0-15 of gestation. Mated females were sacrificed on day 20 of pregnancy. Control animals received the vehicle (0.16% phenol, 0.172% m-cresol and 1.60 % , and 0.19% zinc).

Parameters and endpoints evaluated:**Clinical signs : Daily**

Body weights/food consumptions: Prior to dosing, and twice weekly. Weights were recorded for days 0,3, 6, 8, 12, 14, 16, 18 and day 20 of pregnancy.

Water consumption: Daily for males in weeks -1, 1 and 3, females week 2 and 3.

Plasma Glucose: On day 1 of treatment and again in week 4 at 0, 1, 4 hrs.

Mating Performance: Vaginal smears were taken for 7 days prior to mating.

Terminal examination of Females: Females were sacrificed on day 20 of pregnancy. The ovaries and uteri were examined. The number of corpora lutea, number of implantation sites, number of live/dead fetuses, and number of pre- and post-implantation losses, and fetal/placental abnormalities were recorded. All fetuses were weighed, sexed and examined for external abnormalities. One half of fetuses were examined for visceral abnormalities by the Wilson's technique, the other half were examined for skeletal malformations and variations using alizarin staining by the modified Dawson's technique.

Terminal examination of Males: The males were killed and examined externally and internally for abnormalities, and adrenals, brain, testes, epididymides, vas deferens, prostate, seminal vesicles and pituitary were weighed. Serum motility, sperm morphology and sperm count were examined.

Statistical evaluations: Analysis of incidence of fetal malformations and anomalies were performed using a one-tailed permutation test.

RESULTS:

Mortality and Clinical signs: One male rat (in week 8), one female rat (on day 16 of pregnancy) at 100 U/kg/bid dose of X14, and one male at 100 U/kg/bid of HM(ge) died due to hypoglycemia, histopatholgy showed hemorrhagic depressions/foci in the stomach corpus mucosa in the GI tract. One male which died had hypoglycemic episodes prior to death. No other signs were observed

Body weight: In males, during week 0-4 the body weight gains increased (*p<0.05-0.001) at 25 and 100 U/kg/bid (these were 97%, 108%, 126% and 128% at 5, 25, 100 U/kg/bid of X14 and HM(ge) resp. Similar increases were also noted during week 0-9 in

males (100, 109, 126* and 134*% resp). In females, during week 2-4 the body weight gains increased also at 25 and 100 U/kg/bid (these were 96%, 138%, 158*% and 154*% at 5, 25, 100 U/kg/bid of X14 and HM(ge) resp). Similar increases were also noted during week 2-7 in females (101, 105, 108 and 113% resp). No effects on weight gains were observed at 5 U/kg/bid

Food consumption: In both males and females, food consumptions were increased at mid-high doses (males 96, 102, 112*, and 11*5, females 97, 105, 110*, and 113* at 5, 25, 100 U/kg/bid of X14 and HM(ge) resp). Water consumption was increased in both sexes at mid and high doses during the first week of treatment. — vs — in controls).

Plasma Glucose Concentrations: On day 1 (1-hr) values are shown below. These were all decreased with the drug. However it took more than 4 hrs to recover the blood glucose at high doses (100 U/kg/bid) with both X14 or HM(ge).

Males	females
8.6, 5.2, 5.0, 4.8, 4.8	8.5, 4.2, 5.3, 5.2, 4.3

Mating Performance: The mating performance was not affected, there were no effects on male or female fertility index or copulation index. Pre-coital time (during days 6-10) was slightly higher at mid-high doses (1, 1, 4, and 3 at 0, 5, 25 and 100 U/kg/bid resp), while cell type (sperm, carnified epithelial/epithelial leukocyte) at conception was not significantly altered. Most females conceived within five days of pairing.

FERTILITY IN MALES: Pituitary organ weights in males were significantly decreased ($p < .05-0.01$) in a dose related manner (15.2, 14.9, 13.8*, 13.1*, 14.0 in five groups resp). At 100 U/kg/bid, epididymides weight was slightly lower (— vs — in controls), this was also noted with HM(ge) at this dose. Seminology: This showed a decrease in sperm motility at 100 U/kg/bid (95% of controls). sperm count was lower with HM(ge) (184 vs 202 in controls) but not with the drug (— vs 202 in controls). However, sperm morphology was not significantly different at 100 U/kg/bid with the drug or HM(ge) (93.6 vs 96.6 with controls). Gross pathology in males showed higher incidence of pale subcapsular areas in the liver at 100 U/kg/bid (5-6 animals vs 1 in other groups). Histopathology of testes indicated focal seminiferous epithelial atrophy with vacuolation of sertoli cells (trace-minimal) in 1, 1, 3, 4, and 7 males at 0, 5, 25, 100 of X14 and 100 of HM(ge) U/kg/bid resp. This was due to lower sperm count and motility.

Fertility and Early Embryonic Development in Female rats: Litter values: At 100 u/kg/bid, pre-implantation loss (9.9 vs 6.8 in controls) was higher. Post-implantation losses (12.9 vs 6.7 in controls) were higher also due to increased incidence of early embryonic death, leading to lower litter size and litter weight, Table 9.

Table 9. Litter values in rat segment I/II studies:

	Control	X14			HM(ge)
Dose (U/kg/bid)	0	5	25	100	100
# of Dams	24	23	23	23	24
# of Corpora Lutea	17.9	18.7	18.2	19.1	18.4
# of implantations	16.9	17.4	16.5	17	16.5
Pre-implantation loss (%)	6.8	5.9	8.2	9.9	10.6
# of In utero deaths (early+late)	1.2	1.3	1.3	2.2	2.6*
post-implantation loss (%)	6.7	7.4	9.1	12.9	16.1*
# of live fetuses	15.7	16.2	15.2	14.8	14.0*
Sex ratio (% male)	44.4	47.5	46.4	53.7	48.1
Litter weight (g)	57.5	59.1	55.8	54.2	53.0
Fetal weight (g)	3.7	3.7	3.6	3.6	3.8

*p<.05-0.01

Fetal examinations: Malformations and skeletal and visceral anomalies were observed with all doses of the drug. Small eyes/orbital sockets were observed, 3-4/23 litters were affected in all groups (at 5, 25, 100 and 100 U/kg/bid of X14 and HM(ge) resp), see appendix. Sponsor states that these effects on the fetus have been obtained in previous studies with insulin and are considered to be related to X14 or HM(ge)-induced reduction of maternal blood glucose, rather than the direct effect of the drug on embryofetal development, Table 10.

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Table 10. Malformations and skeletal and visceral anomalies in rat segment I/II toxicity study:

	Control	X14			HM(ge)
Dosage (U/kg/bid)	0	5	25	100	100
Visceral/Skeletal Malformations (FA/LA)	1/1	4/3	4/4	11/5	7/6
Litters examined	24	23	23	23	24
Fetuses examined	376	372	350	341	335
Cranial:					
Small orbital socket	0/0	2/2	3/3	4/3	4/4
Retinal fold	0	0	0	0	1/1
Thoracic					
Interventricular septal defects	0	0	0	2/1	0
Diaphragmatic hernia	0	0	0	1/1	1/1
scoliosis	0	0	0	2/2	0
Lumbar/Abdominal					
incomplete inferior vena cava	0	0	0	2/1	0
Visceral anomalies (FA/LA)	9/8	17/14	22/14	22/15	17/13
Litters examined	24	23	23	23	24
Fetuses examined	189	183	177	165	161
Cranial:					
Dilated lateral ventricle	0/0	0/0	0/0	0/0	1/1
small eye	0	0	1/1	1/1	3/2
Thoracic					
Interventricular septal defects	0	1/1	0	0	2/2
Lumbar/Abdominal					
Thin diaphragm with protrusion liver	0	0	0	3/3	3/3

FA= Fetuses affected, LA= Litters affected

The results indicate that X14 given to male and female rats subcutaneously before the matings, during the matings, and to females on days 0-15 postcoitus, caused anabolic/hypoglycemic effects (all doses reduced blood glucose levels). Two animals died at 100 U/kg/bid due to hypoglycemia (with associated erosion of stomach wall), 25-100 U/kg/bid increased body weight gains (in males by 26-34% and females by 38-58%), food consumptions (by 10-15%), caused testicular changes (focal seminiferous epithelial atrophy with vacuolation of sertoli cells), effects on litters (including pre- and post implantation losses and skeletal and visceral abnormalities (mainly affecting the axial skeleton and eyes). The response in general was similar to that seen with HM(ge). The drug had no effects on the male and female fertility or general reproductive performance of animals. Sponsor states that all the effects, including effects on fetuses, are due to insulin-induced maternal hypoglycemia.

Study title: Segment II Teratology study. Effects of Subcutaneous X14 (Twice Daily) On Embryofetal Development in Rabbits (Study # 940305)

Study No: 940305

Site and testing facility, study initiation-completion: _____

11/15/94-10/21/96.

GLP compliance: Yes

QA- Reports Yes (X) No ():

Lot and batch numbers: X14 06694 — , 06994 , — , HM(ge) 05994

— , 06094 —

METHODS:

Species/strain: New Zealand White female rabbits, \approx 17-26 weeks of age, 2.95-4.25 kg.

Doses employed: 0, 0.5, 1.5 and 5 U/kg bid (or total doses 0, 1, 3 and 10 U/kg/day)

Route of Administration: Subcutaneous, 4-hrs apart

Number of animals/sex/dosing group: 20

Study Design: This study examined the teratogenic effects of X14 in rabbits. Four groups of 20 mated female rabbits were given the drug (at 0.5, 1.5 and 5 U/kg/bid) or recombinant human insulin (HM(ge) at 0.5, 1.5 and 5 U/kg/bid for comparative purposes); subcutaneously twice daily (4-hrs apart), from days 6 to day 18 of gestation. The reason for selecting lower doses here was based on the preliminary study in pregnant rabbits where doses of 6.25, 12.5 and 25 U/kg/bid were administered, which was associated with increased embryofetal deaths (mainly abortions) at all dose levels with highest incidences at 25 U/kg/bid, therefore lower doses were chosen here. Control animals received the vehicle (0.16% phenol, 0.172% m-cresol and 1.60 %, and 0.19% zinc). Surviving females were sacrificed on day 29 PC and necropsied.

Parameters and endpoints evaluated:

Clinical signs : Daily

Body weights/food consumptions: Body weights and food consumptions were recorded on day 0 (post coitum, PC), 2, 6, 8, 10, 14, 19, 23 and day 29 of pregnancy.

Plasma Glucose and TK: On day 6 PC (the first day of treatment), at 0, 1, 4, 5 & 8 hrs. Satellite animals (4/sex/group) were used for TK studies (study # 960419). Plasma samples were assayed for insulin X14 and human insulin.

Terminal examination of Females: Females were sacrificed on day 29 of pregnancy. The ovaries and uteri were examined. The number of corpora lutea, number of implantation sites, number of live/dead fetuses, and number of pre- and post-implantation losses, and fetal/placental abnormalities were recorded. All fetuses were weighed, sexed and examined for external abnormalities. Fetal brains were examined for abnormalities (hydrocephaly, hydroencephaly), and fetuses were examined for skeletal malformations by the modified Dawson's technique.

Statistical evaluations: Analysis of variance followed by intergroup comparisons were performed on BW, food consumptions, litter data and fetal abnormalities. Analysis of

incidence of fetal malformations were also performed using a one-tailed permutation test.

RESULTS:

Mortality and Clinical signs: One control animal was killed (pretreatment) on day 2 PC, following a delivery of 3 dead fetuses, this was replaced with another control animal. One animal was killed on day 25 of pregnancy at 5 U/kg/bid dose of X14 (it had lost weight, it had signs of inappetance and reduced fecal output, it had minimal GI contents, this was a drug related death, and had a plasma glucose levels of 9.0 mmol/l). There were 2 deaths with HM(ge) at 0.5 (not drug related) and 1.5 (on day 7 PC, drug related) U/kg/bid. No other clinical signs were observed.

Body weight: In females, during days 6 to 18 the body weight gains increased at 1.5 and 5 U/kg/bid (these were 212, 221, 338* & 357* g resp in 4 groups of X14, $p < 0.05$ -0.01). These increases generally persisted (compared to controls) till termination day 29. These were also observed with 0.5-5 U/kg/bid of HM(ge) (280*, 283*, 305* resp), but did not persist till day 29.

Food consumption: In females, food consumptions were increased during treatment on days 6-18 at mid-high doses (98, 115*, 122*, 103, 112 and 108 U/kg/bid of X14 and HM(ge) resp), but returned to control values after stopping the treatment with X14, but not with HM(ge) at 5 U/kg/bid.

Plasma Glucose Concentrations: There was a dose related reduction in glucose conc at 1 hr, which returned to normal at 4-hrs, except at 5 U/kg/bid of X14 or HM(ge). The recovery at this dose was noted at 8 hrs with X14 (7.3 vs 7.5 in controls), while it was slower with HM(ge). The reduction in blood glucose was higher with 5 U/kg/bid of HM(ge).

On day 6:	1-hr	4-hrs
with X14:	7.5, 5.4, 5.0, 4.6	7.7, 7.8, 8.0, 5.4
with HM(ge):	5.4, 5.1, 3.9	8.1, 7.4, 5.6

Litter values: At 5 U/kg/bid, there were 2 abortions. At this dose pre-implantation loss (17.6 vs 13.3 in controls) was higher with X14. However it was lower with HM(ge) (7.1 vs 13.3% in controls). Post-implantation losses (34.9 vs 17 in controls) were higher with X14 or HM(ge), due to increased incidence of early embryonic death, leading to lower litter size and litter weight with X14. Fetal weights were not significantly altered. Similarly with 5 U/kg/bid of HM(ge), increase in early embryonic deaths were observed with decrease in litter size and weight, however none of the animals had abortions, Table 11.

Table 11. Litter values in rabbit segment II study:

	Cont	X14			HM(ge)		
Dose (U/kg/bid)	0	0.5	1.5	5.0	0.5	1.5	5.0
# of Dams	18	17	19	17	17	18	20
# of Corpora Lutea	11.1	10.5	11.4	11.2	11.4	11.4	11.0
# of implantations	9.6	9.0	9.9	9.2	10.0	10.6	10.2
Pre-implantation loss (%)	13.3	14.7	12.9	17.6	13.2	6.7	7.1
# of In utero deaths (early+late)	1.5	0.9	1.5	3.3*	1.1	2.1	3.6*
post-implantation loss (%)	17.0	9.5	14.0	34.9*	10.2	19.0	33.7*
# of live fetuses	8.1	8.1	8.4	5.9*	8.9	8.4	6.7*
Sex ratio (% male)	53.6	52.7	48.8	43.6	53.4	46.7	45.5
Litter weight (g)	356	348	382	275*	397	356	302
Fetal weight (g)	46.4	43.6	46.2	48.3	44.9	42.6*	46.4

*p< 0.05-0.01

Fetal examinations: At all doses of X14, but more at 5 U/kg/bid, there was a high proportion of fetuses with skeletal abnormalities affecting the vertebrae, ribs and sternum, these were observed mostly in cervical and thoracic regions, but also in lumbar and caudal regions. These were also noted with 1.5-5 U/kg/bid of HM(ge), Table 12.

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